TRANSMITTAL LETTER TO THE UNITED STATES ATTORNEY'S DOCKET NUMBER 50572

DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371 U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/07370	**31 July 2000**	**9 August 1999**

TITLE OF INVENTION: MONOCELLULAR OR MULTICELLULAR ORGANISMS FOR PRODUCING RIBOFLAVIN

APPLICANT(S) FOR DO/EO/US Henning ALTHOEFER, Oskar ZELDER, Jose L. Revuelta DOVAL, Maria Angeles Santos GARCIA, Hermann SAHM, Klaus-Peter STAHMANN, Ines MAETING

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. /X/ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. / / This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. /X/ This express request to begin national examination procedures (35 U.S.C.371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. /x / A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. /X/ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).

   a./X/ is transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / has been transmitted by the International Bureau.
   c./ / is not required, as the application was filed in the United States Receiving Office (RO/US0).

6. /X/ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. / / Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).

   a./ / are transmitted herewith (required only if not transmitted by the International Bureau).
   b./ / have been transmitted by the International Bureau.
   c./ / have not been made; however, the time limit for making such amendments has NOT expired.
   d./ / have not been made and will not be made.

8. / / A translation of the amendments to the claims under PCT Article 19(35 U.S.C. 371(c)(3)).

9. / X / An oath or declaration of the inventor(s)(35 U.S.C. 171(c)(4)).

10./ / A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11./ X / An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12./ x / An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13./x / A FIRST preliminary amendment.
/ / A SECOND or SUBSEQUENT preliminary amendment.

14./ / A substitute specification.

15./ / A change of power of attorney and/or address letter.

16./x / Other items or information.
International Search Report
International Preliminary Examination Report

[redacted]

10/049265

JC13 Rec'd PCT/PTO 11 FEB 2002

U.S. Appln. No. (If Known)	INTERNATIONAL APPLN. NO. PCT/EP00/07370	ATTORNEY'S DOCKET NO. 50572

17. /X/ The following fees are submitted	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO.....$890.00	**890.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482).....$710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....$740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO .....$ 1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied pro -visions of PCT Article 33(2)-(4).....$100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than / / 20 / /30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

Claims	Number Filed	Number Extra	Rate	
Total Claims	19	-20	X$18.	
Indep.Claims	3	-3	X$84.	
Multiple dependent claim(s)(if applicable)			+280.	
TOTAL OF ABOVE CALCULATION			= **890.**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				
SUBTOTAL			= 890.	
Processing fee of $130. for furnishing the English translation later than / /20 / /30 months from the earliest claimed priority date (37 CFR 1.492(f)).		+		
TOTAL NATIONAL FEE			= **890.**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) $40.00 per property		=	40.	
TOTAL FEES ENCLOSED			= **$ 930.00**	
		Amount to be refunded:	$	
		Charged	$	

a./X/ A check in the amount of $ 930.00 to cover the above fees is enclosed.

b./ / Please charge my Deposit Account No._____in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c./X/ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **11-0345**. A duplicate copy of this sheetis enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

Herbert B Keil
SIGNATURE

Herbert B. Keil
NAME
Registration No. 18,967

SEND ALL CORRESPONDENCE TO:
KEIL & WEINKAUF
1101 Connecticut Ave., N.W.
Washington, D. C. 20036

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of)
)
ALTHOEFER et al.) **BOX PCT**
)
Serial No. 10/049,265)
)
Filed: February 11, 2002)
)

For: MONOCELLULAR OR MULTICELLULAR ORGANISMS FOR PRODUCING RIBOFLAVIN

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on: April 30, 2002
Date of Deposit

Herbert B. Keil
Person Making Deposit

Herbert B Keil
Signature

April 30, 2002
Date of Signature

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS
UNDER 35 USC § 371

Sir:

The following remarks are in reply to the Notification of Missing Requirements dated April 11, 2002.

[redacted]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of
ALTHOEFERet al.

BOX PCT

International Application
PCT/EP 00/07370

Filed: July 31, 2000

For: MONOCELLULAR OR MULTICELLULAR ORGANISMS FOR PRODUCING RIBOFLAVIN

PRELIMINARY AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

Prior to examination, kindly amend the above-identified application as follows:

IN THE CLAIMS

Kindly amend the claims as shown on the attached sheets.

REMARKS

The claims have been amended to eliminate multiple dependency and to place them in better form for U.S. filing. No new matter is included.

A clean copy of the claims is attached.

Favorable action is solicited.

Respectfully submitted,

KEIL & WEINKAUF

/HBKeil

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Reg. No. 18,967

1101 Connecticut Ave., N.W.
Washington, D.C. 20036

(202)659-0100

IN THE SPECIFICATION

Replace the sequence listing in the specification on pages 1/3 to 3/3 with the attached revised sequence listing on pages 1-4.

REMARKS

Translation of the international application into English

The Notification of Missing Requirements stated that the text in the drawings has not been properly translated. Applicants inspected the submitted translated application. Applicants did not find any text in the drawings which has not been properly translated. Attached is a duplicate set of the drawings, which contain no non-English words or phrases.

Number of claims

The Notification of Missing Requirements stated that the number of claims in the International Applications and the number of claims in the translation are not the same. Applicants inspected the amended international application and the translation. Both have 20 claims. The originally filed international application had 21 claims. The internation application was amended at a later stage to contain 20 claims (attached please find the German PCT/IPEA/409, which contains the amended claims 1-20 in German, which corresponds to claims 1-20 as filed with the application). These are the current pending claims.

Processing fee

Applicants do not believe any further fees are necessary since the applicantion as submitted was properly translated.

Sequence Listing

The initially filed application included a Sequence Listing. Applicants herein submit a revised sequence listing including a copy of the computer readable form. The

content of the paper copy of the Sequence Listing and the copy of the Sequence Listing in computer readable form is the same and includes no new matter.

It is believed that by submitting the present amendment and sequence listing diskette, the application now fully complies with the requirements of 37 CFR 1.821-1.825. Favorable action by the examiner is solicited.

Please charge any shortage in fees due in connection with the filing of this paper, including Extension of Time fees to Deposit Account No. 11-0345. Please credit any excess fees to such account.

Respectfully submitted,

KEIL & WEINKAUF

/HBKeil

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Washington, D.C. 20036
(202)659-0100

HBK/DSK/kas

SEQUENCE LISTING

<110> Althoefer, Henning
Zelder, Oskar
Doval, Jose L. Revuelta
Garcia, Maria Angeles Santos
Sahm, Hermann
Stahmann, Klaus-Peter
Maeting, Ines

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CLEAN VERSION OF AMENDED CLAIMS OZ 50572

3. A monocellular or multicellular microorganism as claimed in claim 1, which is a fungus.

4. A monocellular or multicellular microorganism as claimed in claim 1, which is a fungus from the genus Ashbya.

5. A monocellular or multicellular microorganism as claimed in claim 1, which is a fungus of the species Ashbya gossypii.

8. An isocitrate dehydrogenase gene as claimed in claim 6 having an upstream promoter which possesses the nucleotide sequence from nucleotide -661 to -1 as depicted in Fig. 11 (SEQ ID No.1).

9. A gene structure which contains an isocitrate dehydrogenase gene as claimed in claim 6 and also regulatory sequences which are operatively linked to this gene.

10. A vector which contains an isocitrate dehydrogenase gene as claimed in claim 6 or a gene structure which contains an isocitrate dehydrogenase gene.

11. A genetically altered microorganism for the biotechnological production of riboflavin, harboring, in replicatable form, an isocitrate dehydrogenase gene as claimed in claim 6 which is more strongly expressed than in the corresponding microorganism which is not genetically altered and/or whose copy number is increased.

12. A genetically altered microorganism as claimed in claim 11 which harbors a gene structure which contains an isocitrate dehydrogenase gene or a vector which contains an isocitrate dehydrogenase gene.

CLEAN VERSION OF AMENDED CLAIMS OZ 50572

13. A genetically altered microorganism as claimed in claim 11 which harbors an isocitrate dehydrogenase which exhibits a catalytic activity which is increased, and/or an ability to be inhibited by inhibitors which is decreased, as compared with that of the corresponding microorganism which is not genetically altered.

14. A process for the biotechnological production of riboflavin, which comprises using a microorganism as claimed in claim 1.

16. A process as claimed in claim 15, wherein the increase in enzyme activity is achieved by replacing the promoter and/or increasing the gene copy number.

17. A process as claimed in claim 15, wherein the enzymic activity is increased as a result of the catalytic activity of the isocitrate dehydrogenase being increased and/or the ability of the isocitrate dehydrogenase to be inhibited by inhibitors being decreased.

18. (canceled)

19. A process for preparing a microorganism for the biotechnological production of riboflavin, said process comprising using the isocitrate dehydrogenase gene as claimed in claim 6.

20. A process for preparing a microorganism for the biotechnological production of riboflavin, said process comprising using a gene structure or vector which contains an isocitrate dehydrogenase gene as claimed in claim 6.

MARKED VERSION OF AMENDED CLAIMS OZ 50572

20. [The use of a gene structure as claimed in claim 9, or of a vector as claimed in claim 10,] A process for preparing a microorganism for the biotechnological production of riboflavin [as claimed in any of claims 1 to 5 and 11 to 13], said process comprising using a gene structure or vector which contains an isocitrate dehydrogenase gene as claimed in claim 6.

MARKED VERSION OF AMENDED CLAIMS OZ 50572

3. A monocellular or multicellular microorganism as claimed in claim 1 [or 2], which is a fungus.

4. A monocellular or multicellular microorganism as claimed in claim 1 [any of claims 1 to 3], which is a fungus from the genus Ashbya.

5. A monocellular or multicellular microorganism as claimed in claim 1 [any of claims 1 to 4], which is a fungus of the species Ashbya gossypii.

8. An isocitrate dehydrogenase gene as claimed in claim 6 [or 7] having an upstream promoter which possesses the nucleotide sequence from nucleotide -661 to -1 as depicted in Fig. 11 (SEQ ID No.1).

9. A gene structure which contains an isocitrate dehydrogenase gene as claimed in claim 6 [any of claims 6 to 8] and also regulatory sequences which are operatively linked to this gene.

10. A vector which contains an isocitrate dehydrogenase gene as claimed in claim 6 [any of claims 6 to 8] or a gene structure [as claimed in claim 9] which contains an isocitrate dehydrogenase gene.

11. A genetically altered microorganism for the biotechnological production of riboflavin, harboring, in replicatable form, an isocitrate dehydrogenase gene as claimed in claim 6 [any of claims 6 to 8] which is more strongly expressed than in the corresponding microorganism which is not genetically altered and/or whose copy number is increased.

12. A genetically altered microorganism as claimed in claim 11 which harbors a

MARKED VERSION OF AMENDED CLAIMS OZ 50572

gene structure which contains an isocitrate dehydrogenase gene [as claimed in claim 9] or a vector which contains an isocitrate dehydrogenase gene [as claimed in claim 10].

13. A genetically altered microorganism as claimed in claim 11 [or 12] which harbors an isocitrate dehydrogenase which exhibits a catalytic activity which is increased, and/or an ability to be inhibited by inhibitors which is decreased, as compared with that of the corresponding microorganism which is not genetically altered.

14. A process for the biotechnological production of riboflavin, which comprises using a microorganism as claimed in claim 1 [any of claims 1 to 5 or 11 to 13].

16. A process as claimed in claim 15, wherein the increase in enzyme activity is achieved by replacing the promoter and/or increasing the gene copy number.

17. A process as claimed in claim 15 [or 16], wherein the enzymic activity is increased as a result of the catalytic activity of the isocitrate dehydrogenase being increased and/or the ability of the isocitrate dehydrogenase to be inhibited by inhibitors being decreased.

18. (canceled)

19. [The use of an isocitrate dehydrogenase gene as claimed in any of claims 6 to 8] A process for preparing a microorganism [as claimed in any of claims 1 to 5 and 11 to 13] for the biotechnological production of riboflavin, said process comprising using the isocitrate dehydrogenase gene as claimed in claim 6.

MARKED VERSION OF AMENDED CLAIMS OZ 50572

20. [The use of a gene structure as claimed in claim 9, or of a vector as claimed in claim 10,] A process for preparing a microorganism for the biotechnological production of riboflavin [as claimed in any of claims 1 to 5 and 11 to 13], said process comprising using a gene structure or vector which contains an isocitrate dehydrogenase gene as claimed in claim 6.

CLAIMS AS FILED OZ 50572

1. A monocellular or multicellular microorganism for the biotechnological production of riboflavin, which exhibits an activity of an NAD(P)H-forming enzyme which is higher than that of a wild type of the species Ashbya gossypii ATCC 10895.

2. A monocellular or multicellular microorganism as claimed in claim 1, which exhibits an elevated isocitrate dehydrogenase activity.

3. A monocellular or multicellular microorganism as claimed in claim 1, which is a fungus.

4. A monocellular or multicellular microorganism as claimed in claim 1, which is a fungus from the genus Ashbya.

5. A monocellular or multicellular microorganism as claimed in claim 1, which is a fungus of the species Ashbya gossypii.

6. An isocitrate dehydrogenase gene having a nucleotide sequence which encodes the amino acid sequence given in Fig. 11 (SEQ ID No.2) and its allelic variations.

7. An isocitrate dehydrogenase gene as claimed in claim 6 having the nucleotide nucleotide 1 to nucleotide 1262 as depicted in Fig. 11 (SEQ ID No.1) .

8. An isocitrate dehydrogenase gene as claimed in claim 6 having an upstream promoter which possesses the nucleotide sequence from nucleotide -661 to -1 as depicted in Fig. 11 (SEQ ID No.1).

9. A gene structure which contains an isocitrate dehydrogenase gene as claimed in

CLAIMS AS FILED OZ 50572

claim 6 and also regulatory sequences which are operatively linked to this gene.

10. A vector which contains an isocitrate dehydrogenase gene as claimed in claim 6 or a gene structure which contains an isocitrate dehydrogenase gene.

11. A genetically altered microorganism for the biotechnological production of riboflavin, harboring, in replicatable form, an isocitrate dehydrogenase gene as claimed in claim 6 which is more strongly expressed than in the corresponding microorganism which is not genetically altered and/or whose copy number is increased.

12. A genetically altered microorganism as claimed in claim 11 which harbors a gene structure which contains an isocitrate dehydrogenase gene or a vector which contains an isocitrate dehydrogenase gene.

13. A genetically altered microorganism as claimed in claim 11 which harbors an isocitrate dehydrogenase which exhibits a catalytic activity which is increased, and/or an ability to be inhibited by inhibitors which is decreased, as compared with that of the corresponding microorganism which is not genetically altered.

14. A process for the biotechnological production of riboflavin, which comprises using a microorganism as claimed in claim 1.

15. A process for preparing a riboflavin-producing monocellular or multicellular organism, which comprises using recombinant methods to increase the activity of an NAD(P)H-forming enzyme in comparison to that of a wild type of the species Ashbya gossypii ATCC 10895.

CLAIMS AS FILED OZ 50572

16. A process as claimed in claim 15, wherein the increase in enzyme activity is achieved by replacing the promoter and/or increasing the gene copy number.

17. A process as claimed in claim 15, wherein the enzymic activity is increased as a result of the catalytic activity of the isocitrate dehydrogenase being increased and/or the ability of the isocitrate dehydrogenase to be inhibited by inhibitors being decreased.

18. (canceled)

19. A process for preparing a microorganism for the biotechnological production of riboflavin, said process comprising using the isocitrate dehydrogenase gene as claimed in claim 6.

20. A process for preparing a microorganism for the biotechnological production of riboflavin, said process comprsing using a gene structure or vector which contains an isocitrate dehydrogenase gene as claimed in claim 6.

FZJ 9909 PCT 11.13.2000

Forschungszentrum [Research Center] Jülich GmbH
BASF Aktiengesellschaft

Monocellular or multicellular organisms for producing riboflavin

The present invention relates to a monocellular or multicellular organism for producing riboflavin.

Vitamin B_2, also termed riboflavin, is essential for humans and animals. Vitamin B_2 deficiency results in the appearance of inflammation of the oral and pharyingeal mucosae, tears in the corners of the mouth, pruritus and inflammation in the skin folds, and other skin damage, conjunctivitis, diminished visual acuity and clouding of the cornea. Cessation of growth and decrease in weight can occur in infants and children. Vitamin B_2 is therefore of economic importance, in particular as a vitamin preparation in association with vitamin deficiency and as a feed additive. In addition to this, it is also employed as a foodstuff dye, for example in mayonnaise, ice cream, blancmange, etc.

Riboflavin is produced either chemically or microbially. In the chemical methods of production, the riboflavin is as a rule obtained in multistep processes as a pure end-product, with, however, it being necessary also to employ relatively expensive starting materials, such as D-ribose.

The use of microorganisms to produce riboflavin provides an alternative to producing this substance chemically. The microbial production of riboflavin is particularly suitable for use in those cases in which there is no requirement for the substance to be highly pure. This is, for example, the case when the riboflavin is to be used as an additive for animal feed products. In such cases, the microbial production of riboflavin has the advantage that this substance can be obtained in a single-step process. It is also possible

to use renewable raw materials, such as vegetable oils, as starting materials for the microbial synthesis.

The production of riboflavin by fermenting fungi such as Ashbya gossypii or Eremothecium ashbyi has been disclosed (The Merck Index, Windholz et al., eds. Merck & Co., page 1183, 1983, A. Bacher, F. Lingens, Augen. Chem. 1969, p. 393); however, yeasts, such as Candida or Saccharomyces, and bacteria, such as Clostridium, Bacillus and Corynebacterium, are also suitable for producing riboflavin.

Furthermore, methods using the yeast Candida famata have been described, for example, in US 05231007.

Riboflavin-overproducing bacterial strains have been described, for example, in EP 405370, with the strains having been obtained by transforming the riboflavin biosynthesis genes from Bacillus subtilis. However, these prokaryotic genes were unsuitable for a recombinant method for producing riboflavin using eukaryotes such as Saccharomyces cerevisiae or Ashbya gossypii. For this reason, genes which were specific for riboflavin biosynthesis were isolated, according to WO 93/03183, from a eukaryote, namely from Saccharomyces cerevisiae, in order to use them to provide a recombinant method for producing riboflavin in a eukaryotic production organism. However, such recombinant production methods are unsuccessful, or have only limited success, in producing riboflavin if insufficient substrate is provided for the enzymes which are specifically involved in the biosynthesis of the riboflavin.

In 1967, Hanson (Hansom AM, 1967, in Microbial Technology, Peppler, HJ, pp.222-250 New York) found that adding the amino acid glycine increases the formation of riboflavin by Ashbya gossypii. However,

such a method is disadvantageous because glycine is a very expensive raw material. For this reason, efforts were made to optimize the riboflavin production by preparing mutants.

DE 19545468.5 A1 discloses another method for producing riboflavin microbially in which the isocitrate lyase activity, or expression of the isocitrate lyase gene, is increased in a riboflavin-producing microorganism. In addition to this, DE 19840709 A1 discloses a monocellular or multicellular organism, in particular a microorganism, for producing riboflavin by biotechnological means. This organism is distinguished by the fact that it exhibits a glycine metabolism which is altered such that its ability to synthesize riboflavin without any external supply of glycine is at least equal to that of a wild type of the species Ashbya gossypii ATCC10892.

However, even in comparison with these methods, there is still a need for further optimization of riboflavin production.

It is accordingly an object of the present invention to make available a monocellular or multicellular organism, preferably a microorganism, for the biotechnological production of riboflavin, which organism makes it possible to optimize riboflavin formation still further. In particular, it is necessary to make available an organism which makes it possible to achieve a production which is more economic than that of the present state of the art. Above all, the organism should make it possible to achieve a formation of riboflavin which is greater than that achieved by the present organisms.

We have found that this object is achieved by means of a monocellular or multicellular organism whose enzyme

activity with regard to NAD(P)H formation is higher than that of a wild type of the species Ashbya gossypii ATCC10895.

The aim of an accelerated intracellular supply of NAD(P)H can be achieved by increasing the activity of an NAD(P)H-forming enzyme or decreasing the activity of an NAD(P)H-consuming enzyme, or else by altering specificity. This can be achieved using the known methods of the strain improvement of organisms. That is, in the simplest case, the corresponding strains can be prepared in accordance with the selection by means of screening which is customary in microbiology. It is also possible to use mutation with subsequent selection. In this connection, the mutation can be performed either using chemical mutagenesis or using physical mutagenesis. Another method is selection and mutation with subsequent recombination. Finally, the organisms according to the invention can be prepared by means of genetic manipulation.

According to the invention, the organism is altered in such a way that it produces NAD(P)H intracellularly in a quantity which is greater than the organism requires for maintaining its metabolism. According to the invention, this increase in the intracellular production of NAD(P)H can preferably be achieved by preparing an organism in which the enzyme activity of isocitrate dehydrogenase is increased. This can be achieved, for example, by increasing substrate turnover by altering the catalytic center, or else by abolishing the effect of enzyme inhibitors. An increase in the enzymic activity of the isocitrate dehydrogenase can also be brought about by increasing synthesis of the enzyme, for example by means of gene amplification or by eliminating factors which repress enzyme biosynthesis.

According to the invention, the activity of the isocitrate dehydrogenase can preferably be increased by mutating the isocitrate dehydrogenase gene. Such mutations can either be produced in a random manner using classical methods, for example using UV irradiation or mutation-inducing chemicals, or specifically, using recombinant DNA methods, such as deletion, insertion and/or nucleotide exchange.

Isocitrate dehydrogenase gene expression can be achieved by incorporating isocitrate dehydrogenase gene copies and/or by augmenting regulatory factors which exert a positive influence on isocitrate dehydrogenase gene expression. Thus, regulatory elements can preferably be augmented at the transcriptional level by, in particular, increasing transcriptional signals. However, in addition, it is also possible to augment translation by, for example, improving the stability of the mRNA.

In order to increase the gene copy number, the isocitrate dehydrogenase gene can, for example, be incorporated into a gene construct or into a vector which preferably contains regulatory gene sequences which are assigned to the isocitrate dehydrogenase gene, in particular regulatory gene sequences which augment expression of the gene. A riboflavin-producing microorganism is then transformed with the gene construct which contains the isocitrate dehydrogenase gene.

According to the invention, overexpression of the isocitrate dehydrogenase can also be achieved by replacing the promoter. In this connection, it is possible to achieve the higher enzymic activity either by incorporating gene copies or by replacing the promoter. However, it is equally also possible to achieve the desired change in enzyme activity by

simultaneously replacing the promoter and incorporating gene copies.

The change in the isocitrate dehydrogenase gene leads to an accelerated formation of NAD(P)H and, at the same time, to a surprisingly large increase, such as was not previously achievable, in the formation of riboflavin.

The isocitrate dehydrogenase gene is preferably isolated from microorganisms, particularly preferably from fungi. In this connection, preference is once again given to fungi of the genus Ashbya. The species Ashbya gossypii is most highly preferred.

However, all other organisms whose cells contain the sequence for forming isocitrate dehydrogenase, that is plant and animal cells as well, are also suitable for isolating the gene. The gene can be isolated by homologous or heterologous complementation of a mutant which is defective in the isocitrate dehydrogenase gene or else by means of heterologous probing or PCR using heterologous primers. For subcloning, the insert in the complementing plasmid can subsequently be minimized in size by means of suitable steps using restriction enzymes. After sequencing and identification of the putative gene, there then follows a perfect-fit subcloning by means of PCR. Plasmids which carry the resulting fragments as inserts are introduced into the isocitrate dehydrogenase gene-defective mutant, which is tested for the functionality of the isocitrate dehydrogenase gene. Finally, functional constructs are used for transforming a riboflavin producer.

Following isolation and sequencing, the isocitrate dehydrogenase genes can be obtained having nucleotide sequences which encode the given amino acid sequence or its allelic variation. Allelic variations include, in particular, derivatives which can be obtained by

deletion, insertion and substitution of nucleotides from corresponding sequences, with the isocitrate dehydrogenase activity being preserved. A corresponding sequence is given, from nucleotide 1 to nucleotide 1262, in Figure 2b.

A promoter having the nucleotide sequence from nucleotide -661 to -1 as shown in Fig. 11, or a DNA sequence which essentially acts in the same manner, is, in particular, placed upstream of the isocitrate dehydrogenase genes. Thus, a promoter which differs from the promoter having the given nucleotide sequence by means of one or more nucleotide exchanges or by insertion and/or deletion, without, however, the functionality or the activity of the promoter being impaired, can, for example, be placed upstream of the gene. Furthermore, the activity of the promoter can be increased by altering its sequence or the promoter can be completely replaced with active promoters.

Furthermore, regulatory gene sequences or regulatory genes, which, in particular, increase the activity of the isocitrate dehydrogenase gene, can be assigned to the isocitrate dehydrogenase gene. Thus, what are termed "enhancers", which bring about an increase in isocitrate dehydrogenase expression by means of an improved interaction between the RNA polymerase and DNA, can, for example, be assigned to the isocitrate dehydrogenase gene.

One or more DNA sequences can be placed upstream and/or downstream of the isocitrate dehydrogenase gene, with or without a promoter which has been placed upstream, and/or with or without a regulatory gene, such that the gene is contained in a gene structure. By means of cloning the isocitrate dehydrogenase gene, it is possible to obtain plasmids or vectors which contain the isocitrate dehydrogenase gene and which are

suitable for transforming a riboflavin producer. The cells which can be obtained by transformation harbor the gene in replicatable form, i.e. in additional copies on the chromosome, with the gene copies being integrated by means of homologous recombination at arbitrary sites on the genome, and/or on a plasmid or vector.

The monocellular or multicellular organisms which are obtained in accordance with the invention can be any cells which can be employed for biotechnological methods. These cells include, for example, fungi, yeast, bacteria and plant and animal cells. According to the invention, they are preferably transformed cells of fungi, particularly preferably of fungi of the genus Ashbya. The species Ashbya gossypii is particularly preferred in this connection.

The invention is explained in more detail below with the aid of examples without there being any intention of thereby limiting the subject matter to the examples:

The gene for isocitrate dehydrogenase (IDP3) was cloned by PCR and then sequenced (sequence, see Fig. 11). The recombinantly-effected partial deletion of the gene by means of replacement mutagenesis using a geneticin resistance gene (Fig. 1) was confirmed by Southern blotting (Fig. 2). This disruption, i.e. destruction of the gene in the genome of the fungus, results in the fungus no longer being able to form the isocitrate dehydrogenase which is encoded by the gene. Fig. 3 shows the decrease in enzyme activity in the disrupted strain AgΔDP3b as compared with the wild type ATCC 10895. It was possible to demonstrate in preparations of the peroxisomes that this enzyme is located in these organelles (Fig. 10). Whereas the enzyme activity is clearly measurable in wild-type peroxisomes, there is

no longer any activity in the peroxisomes belonging to the disrupted strain.

The disruption of the gene results in a marked decrease in the formation of vitamin as compared with the parent strain (Fig. 4). By contrast, if the gene is introduced, in additional copy and under the control of the strong TEF promoter on a plasmid (Fig. 6), into the Ashbya cells, it is then possible to measure a marked increase in the activity of the enzyme and in the formation of riboflavin (Fig. 5).

Fig. 7 shows that NADPH is required as a reducing agent in the metabolism of unsaturated fatty acids in two out of three alternative reaction pathways. The 2,4-dienoyl-CoA reductase which is involved in this was also found to be located in peroxisomes in Ashbya cells (Fig. 8). Disruption of the IDP3 gene ought now to lead to a decrease in the growth of the cells on linoleic acid or linolenic acid. It was also possible to measure this (Fig. 9). This demonstrates that the importance of IDP3 for the metabolism of the cells lies in the formation of NADPH.

Description of the figures

Fig. 1: Scheme for constructing the vector pIDPkan for replacing the chromosomal *AgIDP3* gene with a gene copy which has been rendered inactive by deletion and insertion of the $G418^R$ gene.

Fig. 2: Use of Southern blotting analysis to check the partial deletion and simultaneous insertion of the geneticin resistance cassette at the *AgIDP* locus. Genomic, *Sph1*-cleaved DNA was hybridized with a digoxygenin-labelled probe.

Fig. 3: Comparison of the enzymic activities of the NADP-specific ICDH from *Ashbya* wild type, from the mutant *A.g. ΔIDP*3b and from the *AgIDP* overexpressors *A.g.* pAGIDP3a and *A.g.* pAGIDP3b during growth on glucose complete medium.

Fig. 4: Comparison of the growth of, and the formation of riboflavin by, the *Ashbya* wild type and the mutant *A.g. ΔIDP*3b during growth on soy-bean oil complete medium.

Fig. 5: Comparison of the growth of, of the formation of riboflavin by, and of the NADP-specific ICDH of, the *Ashbya* wild type and the *AgIDP3* overexpressors *A.g.* pAGIDP3a and *A.g.* pAGIDP3b during culture on soy-bean oil complete medium.

Fig. 6: Plasmid for overexpressing the *AgIDP3* gene under the control of the *TEF* promoter and *TEF* terminator.

In order to introduce the *Sph*l cleavage site, it was necessary to change the nucleotide sequence encoding the second amino acid. This involved a conservative replacement of the amino acid glycine with leucine.

Fig. 7: Catabolic pathways in peroxisomes for unsaturated fatty acids having double bonds at even (A) and odd (B, C) carbon atoms in accordance with Henke *et al.* (1998).

Fig. 8 Separation of organelles isolated from *Ashbya* wild type in a Percoll density gradient:

Activities [U/ml] of the marker enzymes catalase (peroxisomes) and fumarase (mitochondria), of NAD-specific and NADP-specific ICDH and of the 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase which are required for catabolizing unsaturated fatty acids.

Fig. 9: Comparison of the radial growth of *Ashbya* wild type, of the mutants *A.g. ΔIDP*3a and *A.g. ΔIDP*3b and the overexpressors *A.g.* pAGIDP3a and *A.g.* pAGIDP3b on different fatty acids (A: 18:1 cis9; B: 18.2 cis9,12; C: 18:3 cis9,12,15).

Fig. 10: Distribution of the enzymes catalase and ICDH in a Percoll density gradient following centrifugation of organelles from the wild type mycelium (A) and the mutant *A.g. ΔIDP*3b mycelium (B).

Description of the sequence

Nucleotide sequence, and amino acid sequence deduced therefrom, of the *A. gossypii AgIDP3* gene encoding the peroxisomal NADP-specific isocitrate dehydrogenase.

We claim:

1. A monocellular or multicellular microorganism for the biotechnological production of riboflavin, which exhibits an activity of an NAD(P)H-forming enzyme which is higher than that of a wild type of the species Ashbya gossypii ATCC 10895.

2. A monocellular or multicellular microorganism as claimed in claim 1, which exhibits an elevated isocitrate dehydrogenase activity.

3. A monocellular or multicellular microorganism as claimed in claim 1 or 2, which is a fungus.

4. A monocellular or multicellular microorganism as claimed in any of claims 1 to 3, which is a fungus from the genus Ashbya.

5. A monocellular or multicellular microorganism as claimed in any of claims 1 to 4, which is a fungus of the species Ashbya gossypii.

6. An isocitrate dehydrogenase gene having a nucleotide sequence which encodes the amino acid sequence given in Fig. 11 (SEQ ID No. 2) and its allelic variations.

7. An isocitrate dehydrogenase gene as claimed in claim 6 having the nucleotide sequence from nucleotide 1 to nucleotide 1262 as depicted in Fig. 11 (SEQ ID No. 1).

8. An isocitrate dehydrogenase gene as claimed in claim 6 or 7 having an upstream promoter which possesses the nucleotide sequence from nucleotide -661 to -1 as depicted in Fig. 11 (SEQ ID No. 1).

9. A gene structure which contains an isocitrate dehydrogenase gene as claimed in any of claims 6 to 8 and also regulatory sequences which are operatively linked to this gene.

10. A vector which contains an isocitrate dehydrogenase gene as claimed in any of claims 6 to 8 or a gene structure as claimed in claim 9.

11. A genetically altered microorganism for the biotechnological production of riboflavin, harboring, in replicatable form, an isocitrate dehydrogenase gene as claimed in any of claims 6 to 8 which is more strongly expressed than in the corresponding microorganism which is not genetically altered and/or whose copy number is increased.

12. A genetically altered microorganism as claimed in claim 11 which harbors a gene structure as claimed in claim 9 or a vector as claimed in claim 10.

13. A genetically altered microorganism as claimed in claim 11 or 12 which harbors an isocitrate dehydrogenase which exhibits a catalytic activity which is increased, and/or an ability to be inhibited by inhibitors which is decreased, as compared with that of the corresponding microorganism which is not genetically altered.

14. A process for the biotechnological production of riboflavin, which comprises using a microorganism as claimed in any of claims 1 to 5 or 11 to 13.

15. A process for preparing a riboflavin-producing monocellular or multicellular organism, which comprises using recombinant methods to increase

the activity of an NAD(P)H-forming enzyme in comparison to that of a wild type of the species Ashbya gossypii ATCC 10895.

16. A process as claimed in claim 15, wherein the increase in enzyme activity is achieved by replacing the promoter and/or increasing the gene copy number.

17. A process as claimed in claim 15 or 16, wherein the enzymic activity is increased as a result of the catalytic activity of the isocitrate dehydrogenase being increased and/or the ability of the isocitrate dehydrogenase to be inhibited by inhibitors being decreased.

18. The use of a microorganism as claimed in any of claims 1 to 5 and 11 to 13 for producing riboflavin.

19. The use of an isocitrate dehydrogenase gene as claimed in any of claims 6 to 8 for preparing a microorganism as claimed in any of claims 1 to 5 and 11 to 13.

20. The use of a gene structure as claimed in claim 9, or of a vector as claimed in claim 10, for preparing a microorganism as claimed in any of claims 1 to 5 and 11 to 13.

FZJ 9909 PCT

Abstract

The present invention relates to a monocellular or multicellular organism, in particular microorganism, for the biotechnological production of riboflavin, with the enzymic activity of this microorganism with regard to NAD(P)H formation being higher than that of a wild type of the species Ashbya gossypii ATCC10895.

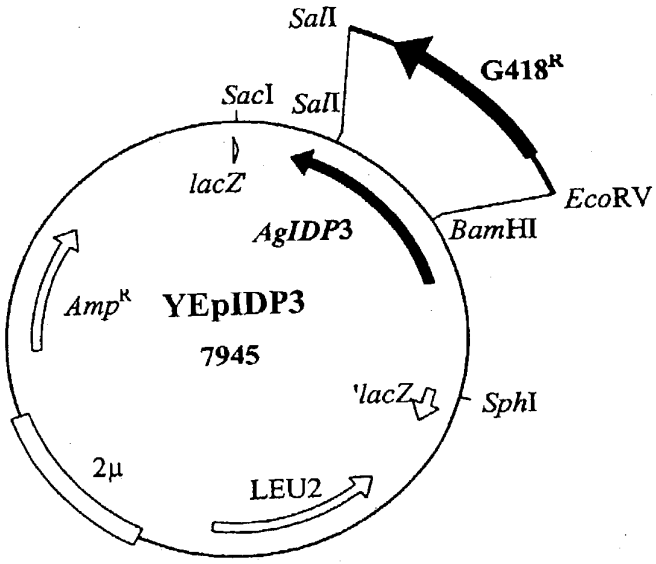

SalI
G418R
SacI
SalI
lacZ'
EcoRV
AgIDP3
BamHI
AmpR
YEpIDP3
7945
'lacZ
SphI
2µ
LEU2

Fig. 1

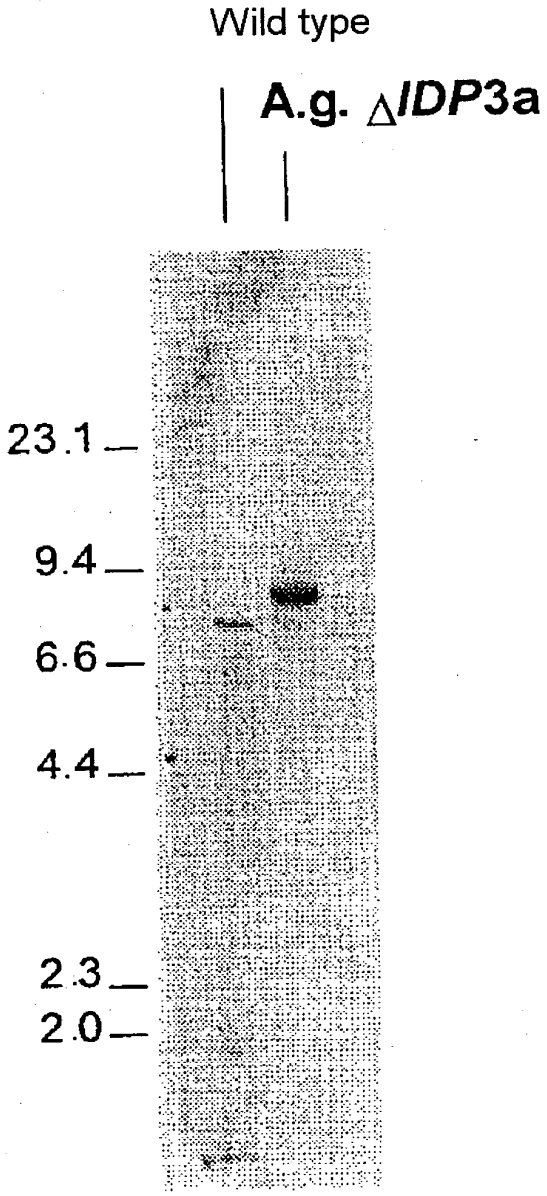


Fig. 2

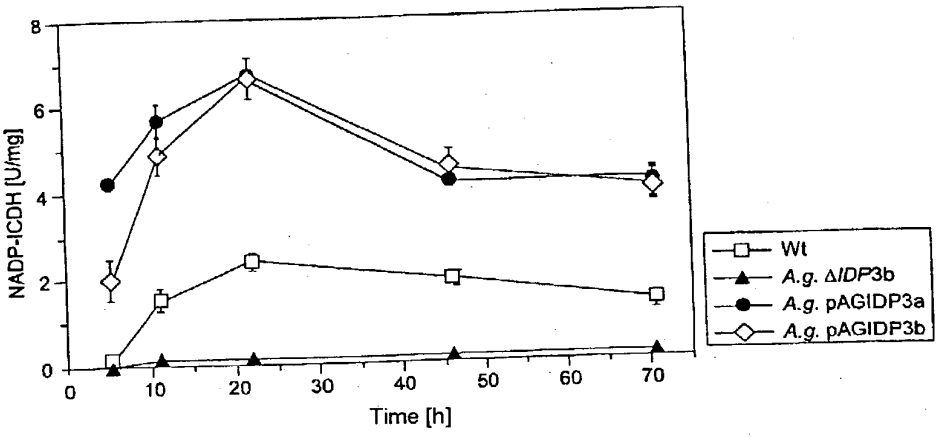

NADP-ICDH [U/mg]
Time [h]
0
2
4
6
8
0
10
20
30
40
50
60
70
Wt
A.g. ΔIDP3b
A.g. pAGIDP3a
A.g. pAGIDP3b

Fig. 3

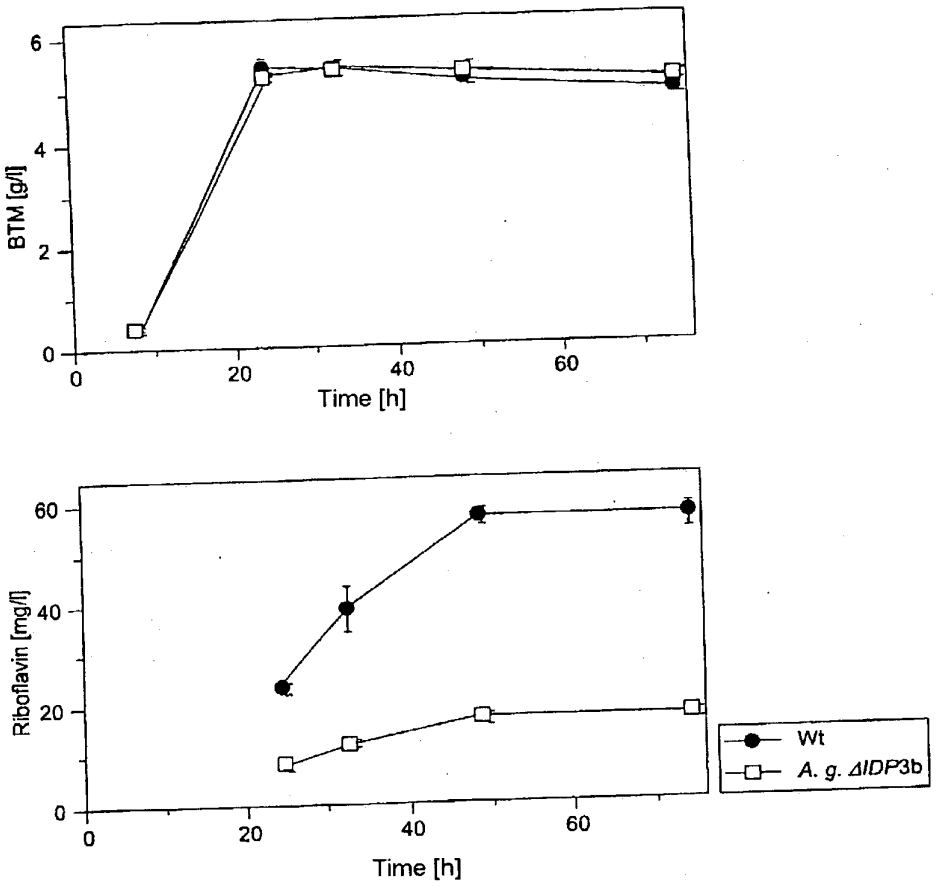


Fig. 4

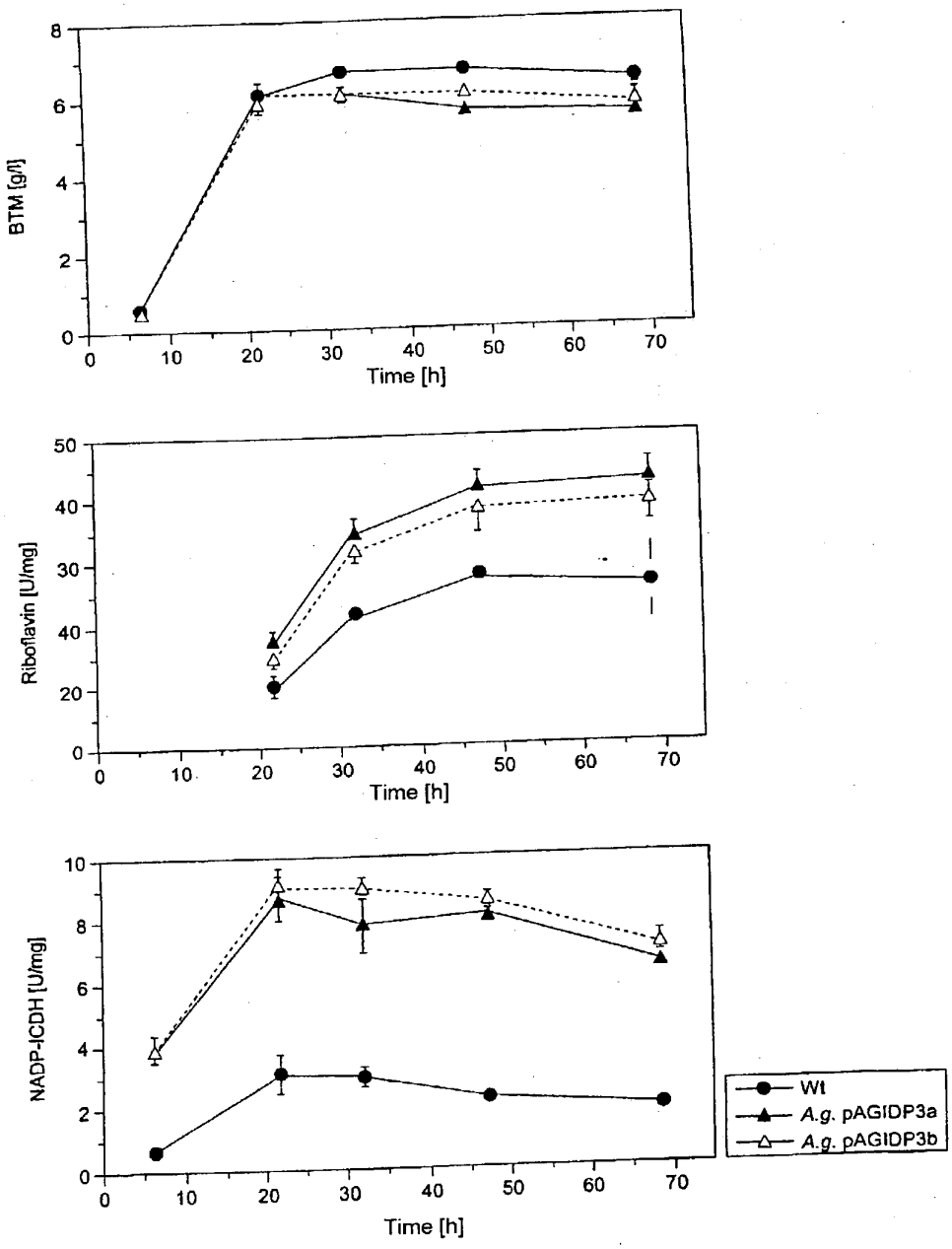
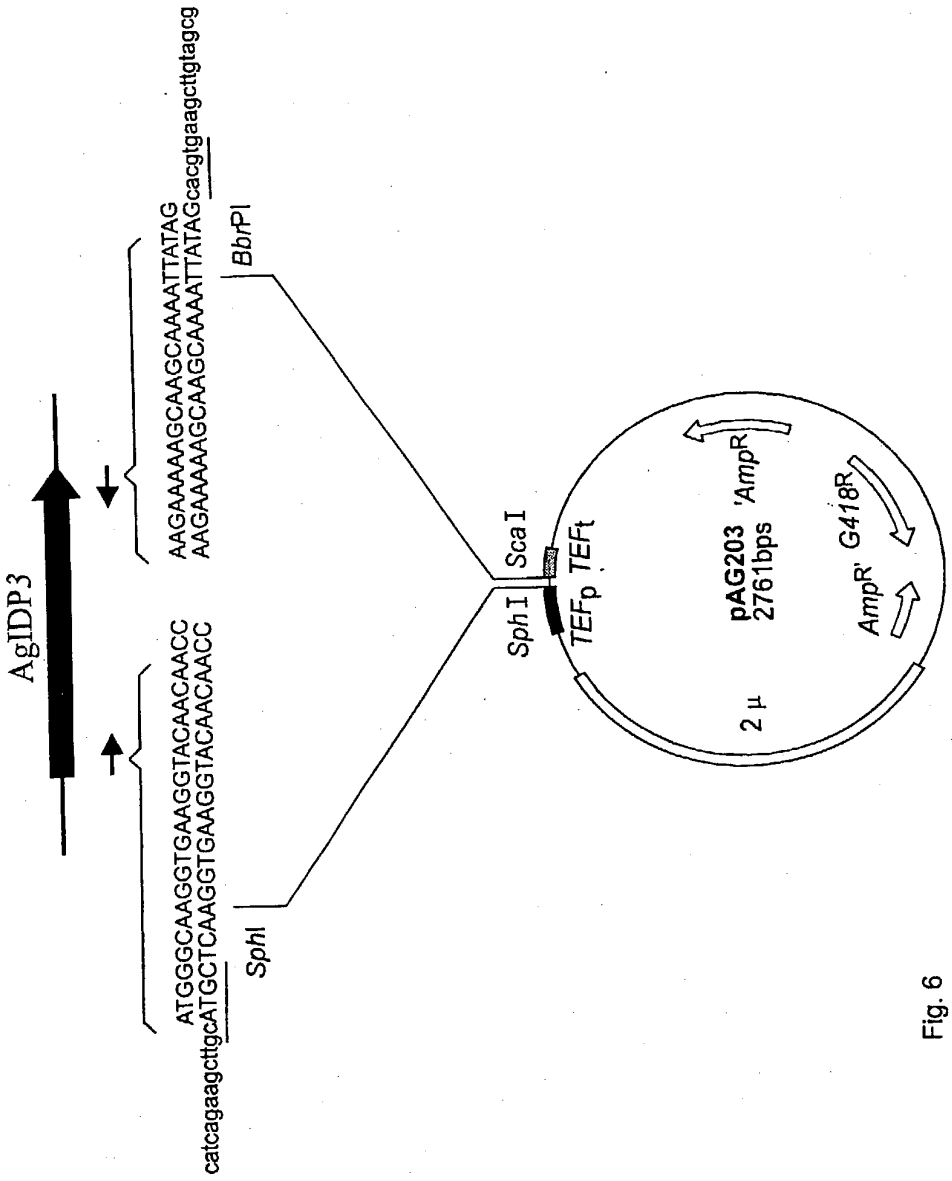

BTM [g/l]
Time [h]
Riboflavin [U/mg]
Time [h]
NADP-ICDH [U/mg]
Time [h]
Wt
A.g. pAGIDP3a
A.g. pAGIDP3b

Fig. 5


AgIDP3
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catcagaagcttgcATGCTCAAGGTGAAGGTACAACAACC
SphI
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AAGAAAAAGCAAGCAAAATTATAGcacgtgaagcttgtagcg
BbrPI
SphI
ScaI
TEFp
TEFt
pAG203
2761bps
2 µ
AmpR'
AmpR
G418R

Fig. 6

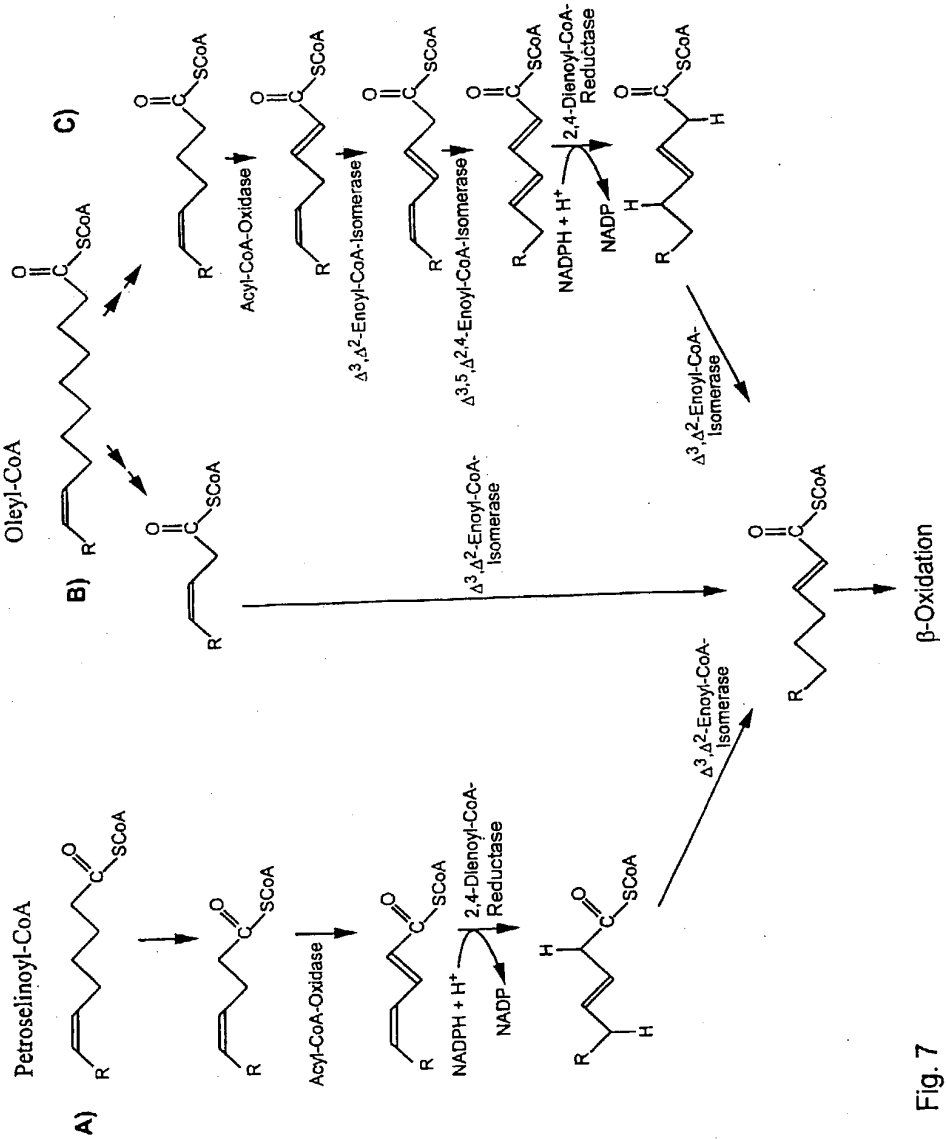

Petroselinoyl-CoA
A)
Acyl-CoA-Oxidase
NADPH + H+
NADP
2,4-Dienoyl-CoA-Reductase
Δ3,Δ2-Enoyl-CoA-Isomerase
Oleyl-CoA
B)
Δ3,Δ2-Enoyl-CoA-Isomerase
C)
Acyl-CoA-Oxidase
Δ3,Δ2-Enoyl-CoA-Isomerase
Δ3,5,Δ2,4-Enoyl-CoA-Isomerase
NADPH + H+
NADP
2,4-Dienoyl-CoA-Reductase
Δ3,Δ2-Enoyl-CoA-Isomerase
β-Oxidation

Fig. 7

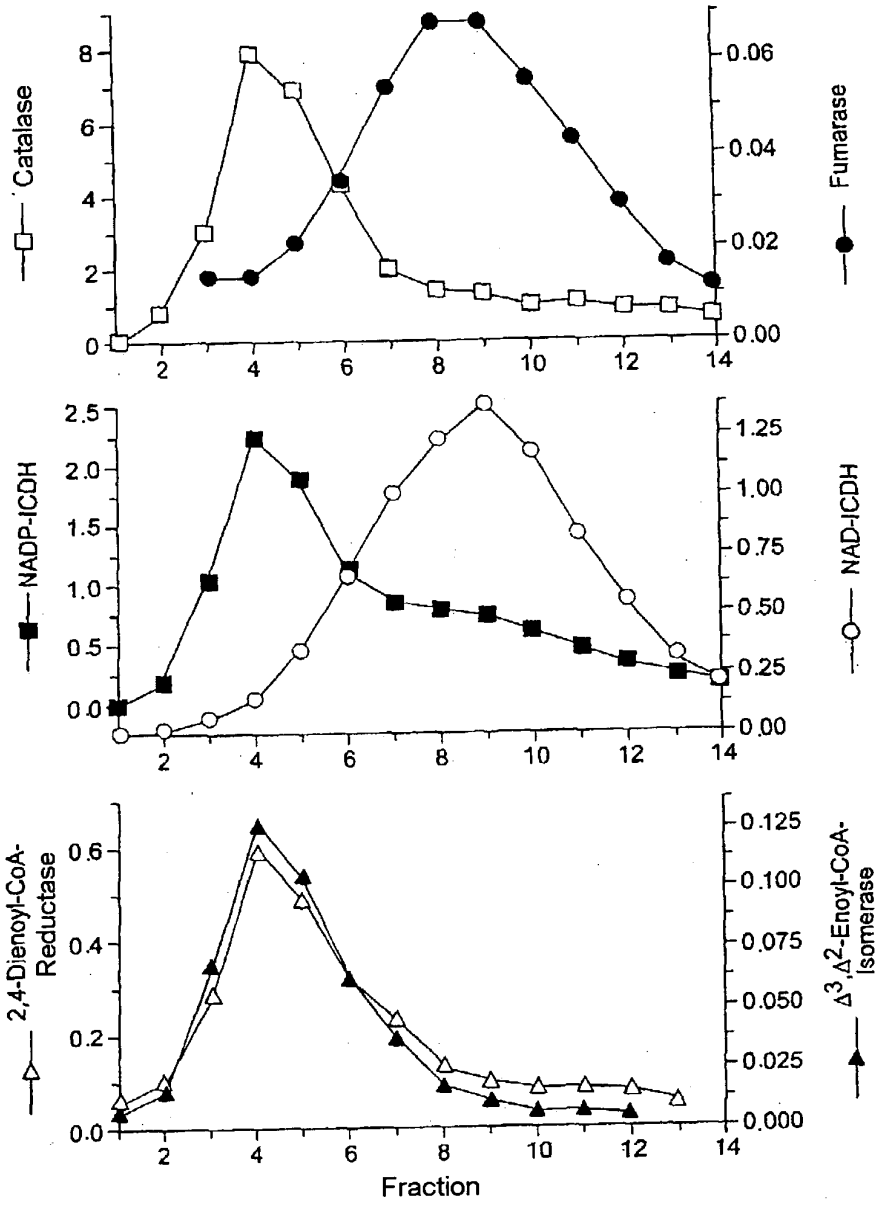
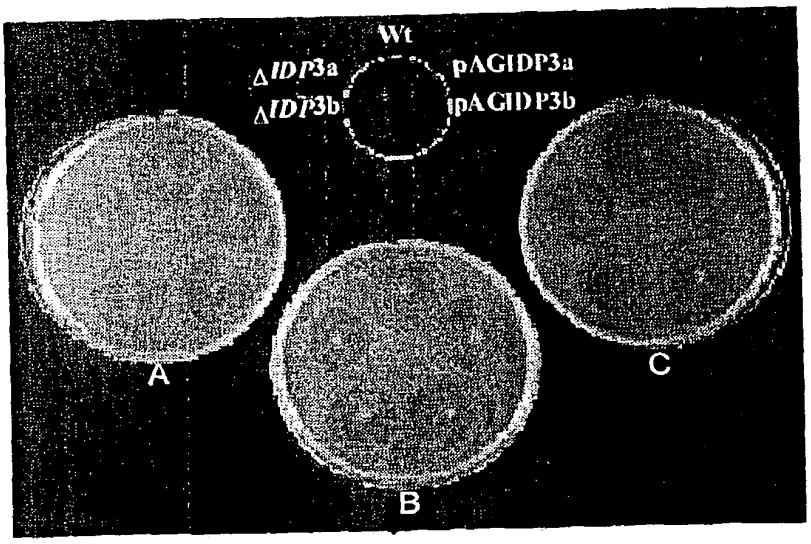

Catalase
Fumarase
NADP-ICDH
NAD-ICDH
2,4-Dienoyl-CoA-Reductase
Δ^3,Δ^2-Enoyl-CoA-Isomerase
Fraction

Fig. 8


Wt
ΔIDP3a
ΔIDP3b
pAGIDP3a
pAGIDP3b
A
B
C

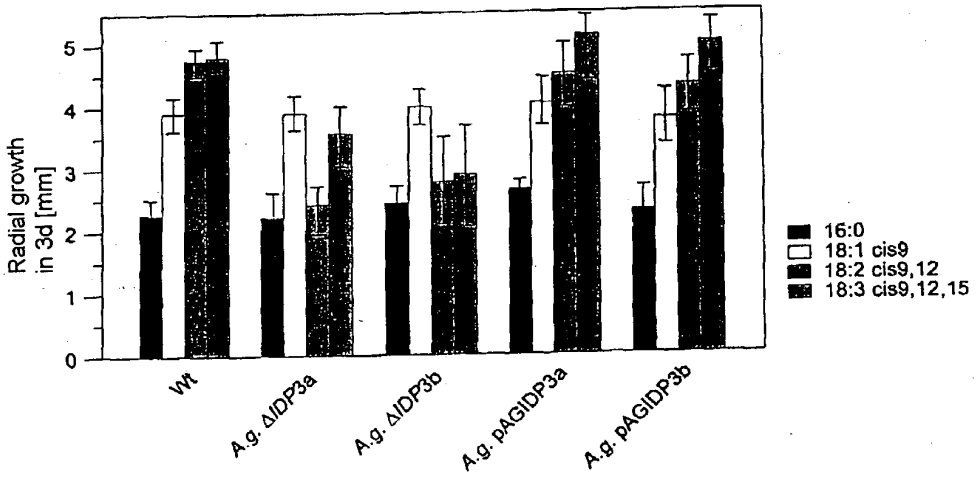

Radial growth in 3d [mm]
0
1
2
3
4
5
Wt
A.g. ΔIDP3a
A.g. ΔIDP3b
A.g. pAGIDP3a
A.g. pAGIDP3b
16:0
18:1 cis9
18:2 cis9,12
18:3 cis9,12,15

Fig. 9

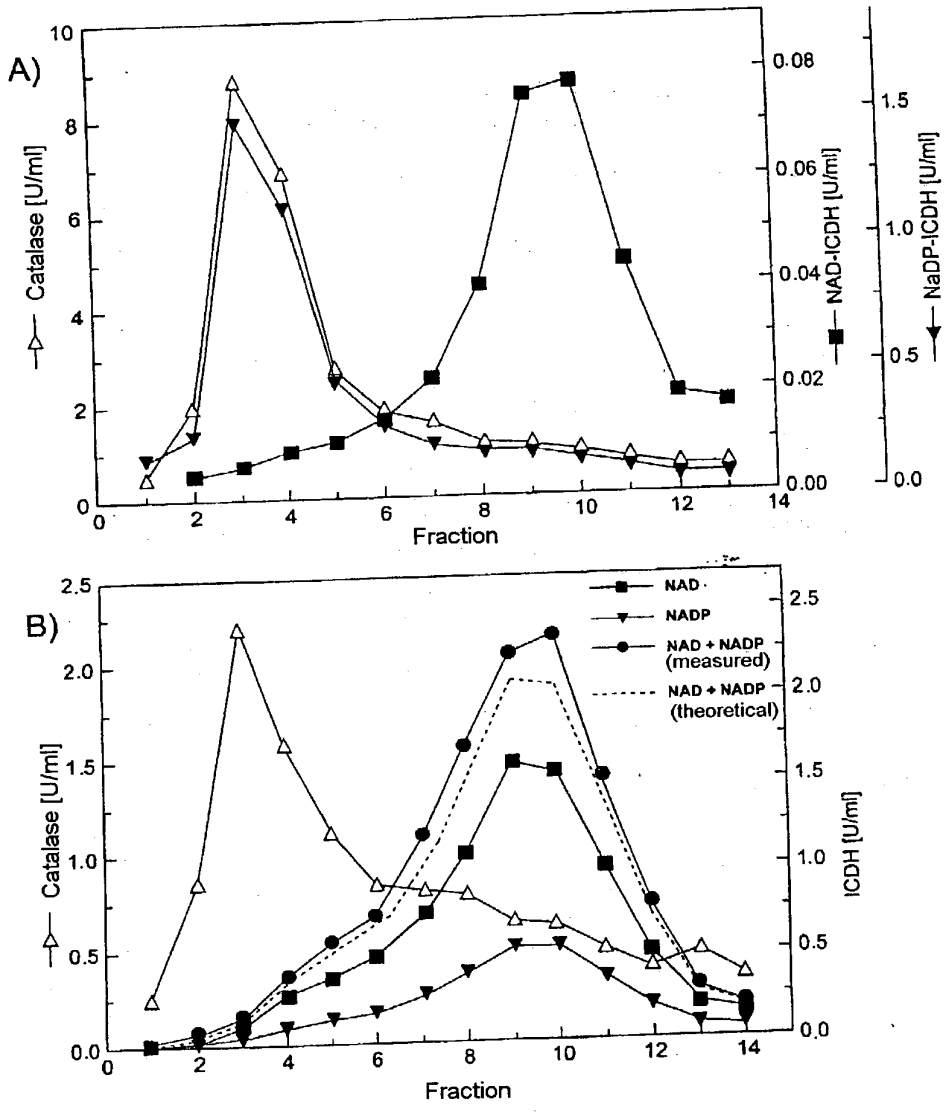

A)
Catalase [U/ml]
NAD-ICDH [U/ml]
NaDP-ICDH [U/ml]
Fraction
B)
NAD
NADP
NAD + NADP (measured)
NAD + NADP (theoretical)
Catalase [U/ml]
ICDH [U/ml]
Fraction

Fig. 10

Declaration, Power of Attorney

0050/050572

We (I), the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Monocellular or multicellular organisms for producing riboflavin

the specification of which

[X] is attached hereto.

[ ] was filed on _____ as

Application Serial No. _____

and amended on _____.

[x] was filed as PCT international application

Number PCT/EP 00/07370

on 31 July 2000

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above–identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)–(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	**Country**	**Day/Month/Year**	**Priority Claimed**
19937548.8	*Germany*	*09 August 1999*	[x] Yes [ ] No

We (I) hereby claim the benefit under Title 35, United States Codes, § 119(e) of any United States provisional application(s) listed below.

_____ (Application Number) _____ (Filing Date)

_____ (Application Number) _____ (Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	**Filing Date**	**Status (pending, patented, abandoned)**

And we (I) hereby appoint **Messrs. HERBERT. B. KEIL**, Registration Number 18,967; and **RUSSEL E. WEINKAUF**, Registration Number 18,495; the address of both being Messrs. Keil & Weinkauf, 1101 Connecticut Ave., N.W., Washington, D.C. 20036 (telephone 202–659–0100), our attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to sign the drawings, to receive the patent, and to transact all business in the Patent Office connected therewith.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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SEQUENCE LISTING

<110> BASF AG, Forschungszentrum [Research Center] Jülich GmbH

<120> Monocellular or multicellular organisms for producing riboflavin

<130> FZJ9909PCT

<140> PCT/EP00/07370
<141> 2000-07-31

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